DOI: 10.1002/chem.200902984

Gramicidin S Derivatives Containing *cis*- and *trans*-Morpholine Amino Acids (MAAs) as Turn Mimetics

Varsha V. Kapoerchan,[b] Emile Spalburg,[c] Albert. J. de Neeling,[c] Roos H. Mars-Groenendijk,[d] Daan Noort,[d] José M. Otero,[e] Patricia Ferraces-Casais,[e] Antonio L. Llamas-Saiz,[f] Mark J. van Raaij,[e, g] Joop van Doorn,[h] Gijs A. van der Marel,[b] Herman S. Overkleeft,[b] and Mark Overhand*[a]

Abstract: The cyclic decapeptide gramicidin S (GS) was used as a model for the evaluation of four turn mimetics. For this purpose, one of the D-Phe-Pro two-residue turn motifs in the rigid cyclic β-hairpin structure of GS was replaced with morpholine amino acids (MAA **2**–**5**), differing in stereochemistry and length of the side-chain. The conformational properties of the thus obtained GS analogues (**6**–**9**) was assessed by using NMR spectroscopy and X-ray crystallography, and correlated with their biological properties (antimicrobial and hemolytic activity). We show that compound **8**, containing the dipeptide isostere *trans*-MAA **4**, has an apparent high structural resemblance with GS and that its antibacterial activity against a panel of Gram positive and -negative bacterial strains is better than the derivatives **6**, **7** and **9**.

Keywords: antibiotics · conformation analysis · gramicidin S · peptides · peptidomimetics

Introduction

The cyclic decapeptide gramicidin S (GS, **1**, cyclo-(Pro-Val-Orn-Leu-D-Phe)$_2$, Figure 1a), produced by *Bacillus brevis*,[1] displays potent antibacterial activity against both Gram-positive and to a lesser extent Gram-negative bacteria, but is toxic to human red blood cells (hemolytic activity).[2] GS kills bacteria by disrupting the bacterial cell membrane.[3] This property is attributed[4] to both the basic and amphiphilic nature of the rigid cyclic β-hairpin molecular structure

[a] Dr. M. Overhand
Bioorganic Synthesis
Leiden Institute of Chemistry, Leiden University
P.O. Box 9502, 2300 RA Leiden (The Netherlands)
E-mail: overhand@chem.leidenuniv.nl

[b] Dr. V. V. Kapoerchan, Prof. Dr. G. A. van der Marel, Prof. Dr. H. S. Overkleeft
Bioorganic Synthesis, Leiden Institute of Chemistry
Leiden University, P.O. Box 9502
2300 RA Leiden (The Netherlands)

[c] E. Spalburg, Dr. A. J. de Neeling
Laboratory for Infectious Diseases
National Institute of Public Health and the Environment
P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

[d] R. H. Mars-Groenendijk, Dr. D. Noort
Defense, Security and SafetyTNO, P.O. Box 45
2280 AA Rijswijk (The Netherlands)

[e] J. M. Otero, Dr. P. Ferraces-Casais, Dr. M. J. van Raaij
Departamento de Bioquímica y Biología Molecular
Faculta de Farmacia Campus Sur
Universidad de Santiago de Compostela
15782 Santiago de Compostela (Spain)

[f] Dr. A. L. Llamas-Saiz
Unidad de Rayos X (RIAIDT)
Laboratorio Integral de dinámica y Estructura de Biomoléculas "José R. Carracido"
Edificio CACTUS, Campus Sur
Universidad de Santiago de Compostela
15782 Santiago de Compostela (Spain)

[g] Dr. M. J. van Raaij
Departamento de Biología Estructural
Instituto de Biología Molecular de Barcelona
Consejo Superior de Investigaciones Científicas, Baldiri Reixac 10
08028 Barcelona (Spain)

[h] Dr. J. van Doorn
Applied Plant Research, P.O. Box 85
2160 AB Lisse (The Netherlands)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200902984.

of GS[5–8] that is stabilized by four intramolecular hydrogen-bonding interactions formed between the C=O and NH of the amides of opposing Val and Leu residues (Figure 1b). The side chains of the two Orn residues are on one side of the molecule and the hydrophobic side chains of the two Val and Leu residues reside on the opposing face, making the molecule amphiphilic (Figure 1c).

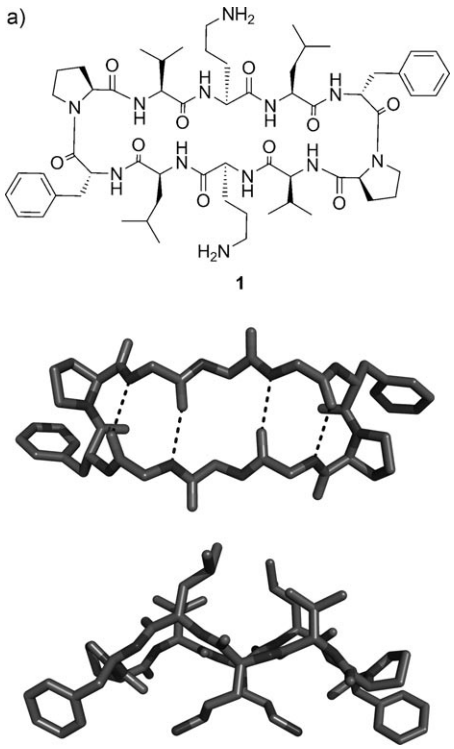

Figure 1. Structure of GS. a) Structural formula of the GS molecule. b) Top view of the GS molecule (Val, Orn and Leu side chains omitted). Hydrogen bonds (stabilizing the secondary structure) are depicted by thin green lines. c) Side view of the GS molecule. Hydrophobic side chains of the Val and Leu residues reside on one face of the molecule, the positively charged side-chains of the two Orn residues are on the opposing face, providing its amphiphilic characteristics.[22]

Because of its well-defined secondary structure, GS has served as a model to probe the effect of turn mimetics on the β-cyclic hairpin structure. The D-Phe-Pro sequence of GS has been replaced by bicyclic thioindolizine derivatives[9,10] as well as the structurally related 5,6-fused azabicycloalkane[11] and indolizines.[12,13] In a related approach, several sugar amino acids (SAA) have been incorporated and their structural and biological properties studied.[14–16] C-functionalized morpholines are found frequently both in natural products and in drugs.[17] Various synthetic strategies towards these compounds have been developed, allowing to obtain morpholines with different substitution patterns. Because of its rigid structure, a morpholine ring incorporated in a GS molecule can possibly invoke structural changes which may affect the antibacterial and hemolytic properties. Here, four GS analogues are presented in which one of the D-Phe-Pro turn motifs is replaced by a *cis*- or *trans*-, δ- or ε-morpholine amino acid (MAA). Their synthesis, structural and biological (antibacterial and hemolytic) properties are evaluated and compared to those of GS.

Results and Discussion

Synthesis of the target compounds: The synthesis of MAAs **2**, **3** and **4** as their azide derivatives was reported previously by us (Figure 2).[18] The synthesis of the novel MAA **5**, as its azide derivative, is described in the Supporting Information. MAAs **2** and **4** represent dipeptide isosteres (δ-amino acids), MAAs **3** and **5** are one carbon atom longer (ε-amino acids). The difference between MAAs **2** and **3** and MAAs **4** and **5** concerns the *cis* or *trans* relationship between the aminomethyl and carboxyl groups of the morpholino ring, respectively. All four MAAs have an *N*-benzyl functionality to mimic the hydrophobic characteristics of the D-Phe-Pro sequence.

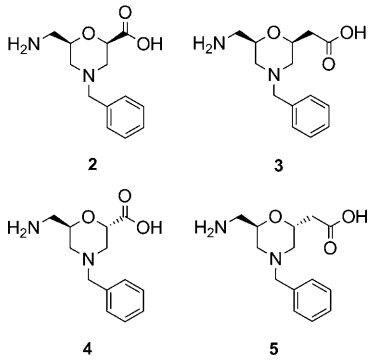

Figure 2. Morphilino amino acids (MAAs) investigated in this study as replacement of one of the type two β-turns in gramicidin S. MAAs **2** and **3** are *cis* configured and MAAs **4** and **5** are *trans* configured δ- and ε-morpholine amino acids, respectively.

The peptides were assembled by adaptation of a standard Fmoc SPPS method, starting with preloaded Fmoc-Leu-HMBP-BHA resin. In the final stage of the syntheses compounds **2**–**5** were incorporated as terminal residues as their azide derivatives. Reduction of the azide functionality[18] was followed by mildly acidic cleavage from the resin. Cyclization with PyBOP, HOBt and DIPEA as base, deprotection of the Boc protecting groups with strong acid and subsequent HPLC purification was straightforward, yielding peptide **6**–**9** (Figure 3) in an overall yield of 25–65%, as their TFA salts.

Structural analysis of synthesized GS analogues 6–9: The effect on the incorporation of the MAAs on the cyclic β-hairpin secondary structure was determined by using 1D and 2D NMR techniques and X-ray crystal structure determination. The observed $J_{NH-H\alpha}$ values of the residues of compounds **6**–**9** were compared to those of GS (Figure 4). In the amide region of the ^{1}H NMR spectra eight signals were observed for all analogues, with the exception of com-

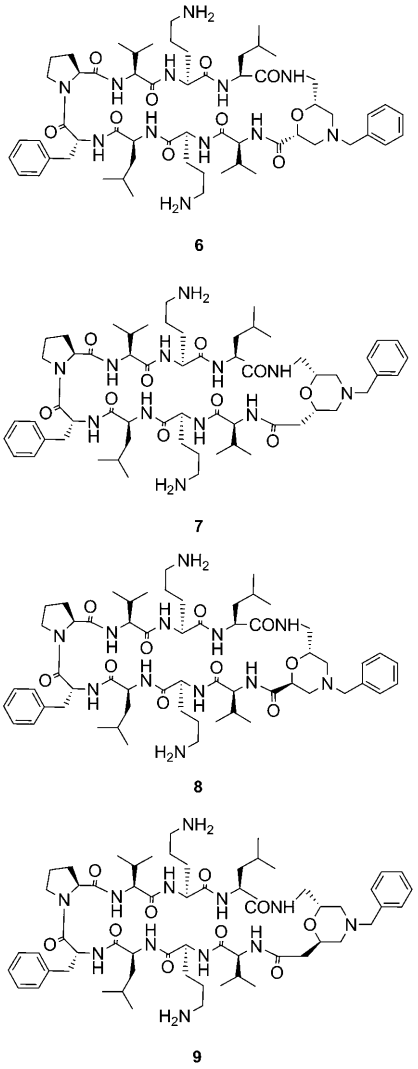

Figure 3. GS analogues containing the morpholine-amino acid residues.

pound **9**. Certain amide signals in the ^{1}H spectrum of **9** were observed as broad singlets instead of doublets and the amide signal of the MAA residue was not observed at all, indicating structural flexibility in this region of the molecule. The $J_{NH-H\alpha}$ values of the ornithine, leucine and valine residues were all between 8 and 12 Hz, which is a strong indication of a β-strand conformation.[19] The $J_{NH-H\alpha}$ values of the D-Phe residues were in the range of 2–4 Hz, which is typical for an amino acid that is part of a β-turn.[19]

These features indicate that the cyclic peptides **6–8** and to a lesser extent compound **9**, adopt overall secondary structures similar to that of GS. A second NMR analysis technique compares the chemical shift of the α-proton of an amino acid residue to the average value of this residue as part of a random coil. The results of this chemical shift perturbation analysis method[20,21] are depicted in Figure 5. The valine, ornithine and leucine residues show positive perturbations indicating that these residues are part of a β-strand and all proline and D-phenylalanine residues display negative perturbations, consistent with their presence in a turn.

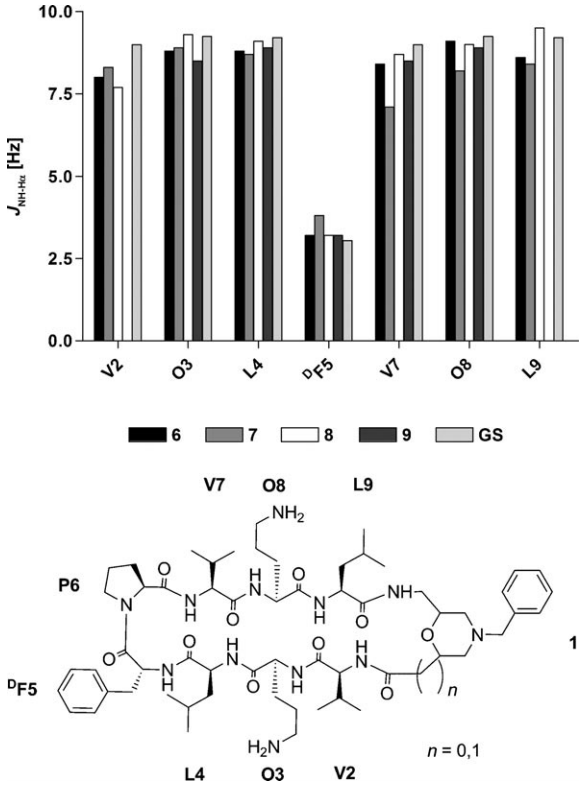

Figure 4. Comparison of $J_{NH-H\alpha}$ values of the analogues **6–9** to those of GS. In analogue **9**, the CONH signals of the V2 and L9 residues were observed as broad signals, so no coupling constant could be determined. The $J_{NH-H\alpha}$ values for analogue **7** were taken from ref. [18].

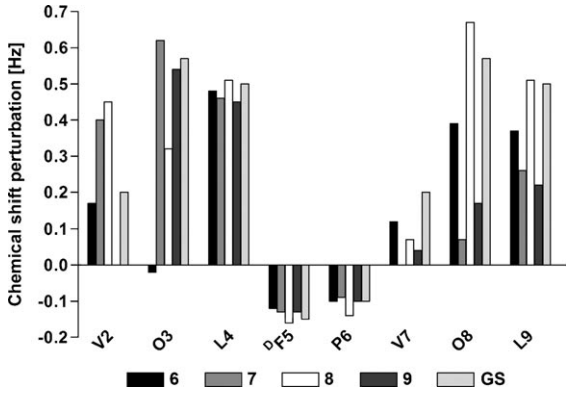

Figure 5. Chemical shift perturbation of H_α protons in GS analogues **6–9** as compared to GS. For ornithine residues, the reported[20] $\delta(H_\alpha)$ random coil value of lysine was taken. The chemical shift perturbation was calculated as $\Delta\delta H_\alpha = \delta H_\alpha(\text{observed}) - \delta H_\alpha(\text{random coil})$. For the V2 residue of **9** no difference between the observed and reported value was seen. The H_α of V7 in **7** was not observed.

Overall, the chemical shift perturbation indicates a cyclic β-hairpin-like secondary structure. However, specific differences in perturbation values are found when the analogues are compared to GS.

To obtain detailed molecular information nuclear Overhauser effect (NOE) experiments were performed for all an-

alogues at 298 K. (see Figure S1 in the Supporting Information). Several sequential NOEs are observed in the NH region of the 2D spectra of compounds **6–9**, however, the observation of specific long-range NOEs in the turn regions are crucial. For peptide **6**, a long range NOE crosspeak between Val2-NH and Leu9-NH (peak B in Figure S1, panel I) is observed. This implies that these NH protons are in close proximity to each other. As such correlations are also found for GS itself, the presence of this NOE crosspeak in **6** may indicate a conformation similar to that of GS. Interestingly, in the NOE spectrum of compound **8** (the *trans* isomer of compound **6**) also this long-range NOE crosspeak between Val2-NH and Leu9-NH is observed (B, Figure S1, panel III).

Two long-range crosspeaks in the turn region between Val2-NH and Leu9-NH and between MAA-NH and Val2-NH of peptide **7** indicates an altered turn conformation. Peptide **9** did not show NOE crosspeaks around the MAA turn, in agreement with partial flexibility in this region as observed in the 1D spectra.

Crystallization trials were performed with analogues **6–9**, only yielding suitable crystals for X-ray analysis of peptides **7** and **8**. Peptide **7** adopts overall a similar secondary structure in the crystal as GS, with a distorted β-turn conformation. As a consequence, only three instead of the usual four intramolecular hydrogen-bonding interaction are observed, due to the distorted orientation ("flip" of the amine bond)[14,16] connection of this MAA residue with the neighboring Leu9 residue (Figure 6a). This altered turn region of compound **7** is in agreement with the findings of the NMR experiments. Interestingly, the cyclic β-hairpin secondary structure as observed in the X-ray structure of compound **8** is very similar to that of GS (Figure 6b), with all four intramolecular hydrogen-bonds in place. This X-ray structure of **8** is in agreement with the NMR data. (See Figure S2 for X-ray assembly of compound **8**).

Evaluation of biological characteristics: GS analogues **6–9** were assayed against a panel of Gram-positive and Gram-negative bacterial strains. The results are shown in Table 1.

As can be seen, none of the four analogues are significantly more potent than GS. Peptides **6** and **7** show decreased inhibitory activity for all Gram-positive bacteria tested, especially for *E. faecalis*. Compound **8** performs almost as well as GS, with the exception of the *E. faecalis* and the *S. epidermis* strains. Peptide **9** has similar antimicrobial activity as the compounds **6** and **7**. Peptides **6–9** were also evaluated for their toxicity towards human red blood cells (Figure 7). All peptides are less hemolytic when compared to GS, with **8** displaying the most hemolytic activity and **6** being the least toxic.

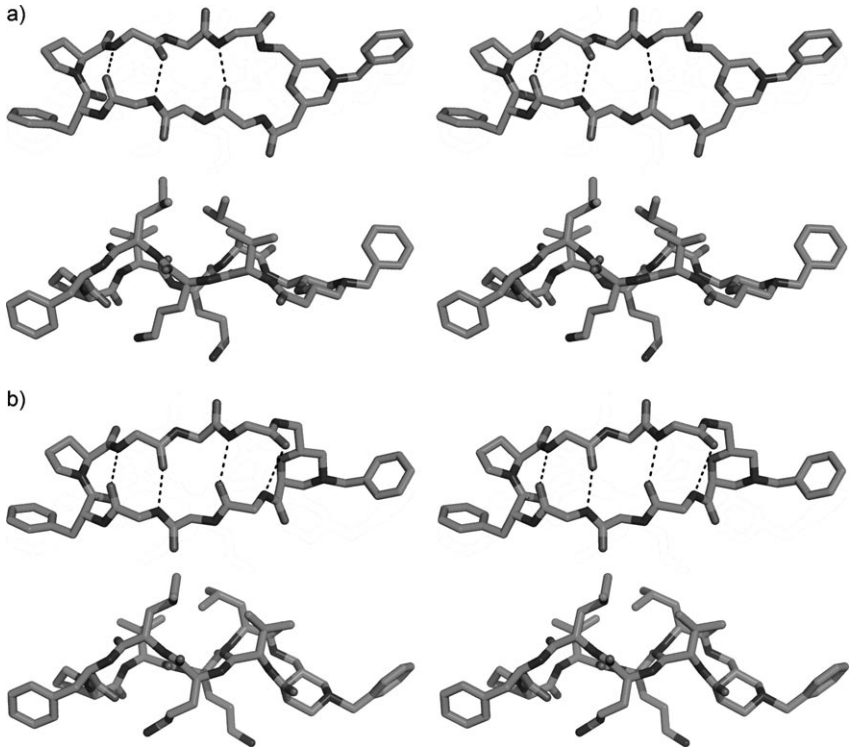

Figure 6. a) X-ray analysis of peptide **7**: stereoview of the peptide monomer. In the top view, side chains are omitted for clarity. Intramolecular hydrogen-bonds are indicated with dotted lines. b) Top and side stereoview of the monomer of **8**.

Table 1. Antibacterial activity of compounds **6–9** as compared to GS. MIC values (MIC = minimal inhibitory concentration) are given in μg per mL and were measured after 24 h of incubation. The experiments were conducted once. The experimental error is one MIC interval.

	Gram+ *S. aureus*	Gram+ *S. epidermidis*	Gram+ *E. faecalis*	Gram+ *B. cereus*	Gram− *E. coli*	Gram− *aeruginosa*
GS	8	4	8	8	32	64
6	16	16	64	16	64	>64
7	16	8	64	16	64	>64
8	8	8	16	8	32	64
9	32	8	64	16	64	>64

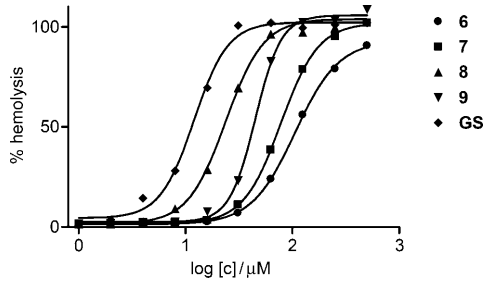

Figure 7. Hemolytic activity of GS analogues **6**–**9**. Experiments were carried out in duplo. Multiple blood donors (both male and female) were used. A maximum of 10% experimental error was found.

Discussion and Conclusion

In this work, four GS analogues **6**–**9** are presented, in which a D-Phe-Pro motif of one of the β-turns was replaced with *cis*- and *trans*, δ- and ε-morpholine amino acids (MAAs **2**–**5**). The secondary structures of these analogues and their biological properties (antibacterial and hemolytic activity) were evaluated and compared with those of GS.

Compound **7** adopts a distorted β-turn as result of the presence of the *cis* ε-MAA **3**. This distortion is characterized by a repositioning of one of the turn amides (amide flip) in such a way that no intra-molecular hydrogen-bonding interaction is possible (see Figure 6a). A similar distorted β-turn arrangement has been observed by us in a series of related GS analogues containing sugar amino acids (SAAs).[14,16] The β-turn of compound **9**, containing the *trans* ε-MAA **5**, is flexible. The isomeric compounds **7** and **9** have a comparable biological profile.

The macrocycles **6** and **8**, containing *cis* δ-MAA **2** and *trans* δ-MAA **4**, respectively, both agree well with the cyclic β-hairpin secondary structural characteristics of GS. In case of compound **6** this is the expected outcome since we and others have designed and synthesized related turn mimetics, such as sugar amino acids (SAAs), having *cis* configured aminomethyl- and carboxyl groups functionalities.[23–37] However, in general *trans* isomers are considered as mimetics of extended conformations and we were thus somewhat surprised to observe that also compound **8** assumes a secondary structure strongly resembling that of GS, with all four intramolecular hydrogen bonds apparently in place. It can be assumed that the octapeptide sequence originating from GS overrules a possible intrinsic conformational preference that MAA **4** would transpose to a linear oligopeptide.

The X-ray structure of compound **8** overlaid with GS is depicted in Figure 8. Comparison of the distance information with respect to the turn region of both compounds (Table 2) reveals that the determined distance between Val2-NH···O=Leu9 is more than 1 Å longer than the corresponding distance in GS and the N-H···O angle is very small (122.7 versus 161.5°). Whether this is considered a hydrogen bond is therefore doubtful[38] but fact is that the Val2-NH···O=Leu9 functionalities are rigidly positioned in close proximity in compound **8**. Therefore, in spite of the lack of an intramolecular hydrogen bond in the MAA-containing turn region of **8** the geometry of the two peptidic bonds involved in the turn is quite similar to the usual β-turn motive in GS.

Table 2. Structural data extracted from the crystal structures from GS and compound **8**.

	GS[39]	Peptide **8**
Leu9 O···H-N Val2	2.15 Å	3.31 Å
Leu9 O···N Val2	2.99 Å	3.86 Å
angle O···H-N	161.5°	122.7°

The positioning of the *N*-benzyl group of the MAA residue is different in comparison with the benzyl side-chain of D-Phe residue of GS (Figure 8). Interestingly, compound **8** is a much better antimicrobial agent than compound **6**, with comparable activity as GS and slightly less hemolytic.

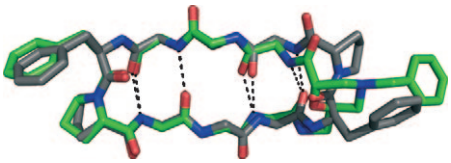

Figure 8. X-ray structure of GS (white) overlayed to compound **8** (green). Intramolecular hydrogen-bonds are indicated with dotted lines in red for GS and black for **8**. Side chains are omitted for clarity.

In conclusion, we have demonstrated that morpholine amino acids are useful building blocks in the design of new amphiphilic cationic cyclic peptides. Specifically, GS analogue **8**, incorporating *trans*-configured **4**, most closely resembles GS itself both in secondary structure and biological activity. Compound **8** has antibacterial activity comparable to that of GS and is less hemolytic. From a structural point this finding is interesting, since related *trans*-substituted dipeptide isosteres are reputed to induce linear configurations.[23,35–37] With respect to the identification of GS derivatives that are bactericidal with reduced hemolytic activity that a strategy in which the morpholine secondary amine is alkylated or acylated with a range of lipophilic functionalities is well worth pursuing.

Experimental Section

Antibacterial assays: The following bacterial strains were used: *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). Bacteria were stored at −70°C and grown at 30°C on Columbia Agar with sheep blood (Oxoid, Wesel, Germany) suspended in physiological saline until an optical density of 0.1 AU (at 595 nm, 1 cm cuvette). The suspension was diluted (10×) with physiological saline, and 2 μL of this inoculum was added to 100 μL growth medium, Nutrient Broth from Difco (ref. nr. 234000, lot nr. 6194895) with yeast extract (Oxoid LP

Table 3. Selected crystallographic data of the crystals obtained from **7** and **8**.

	7	**8**
formula	$2(C_{60}H_{97}N_{12}O_{10})\cdot O_{17}$	$2(C_{59}H_{95}N_{12}O_{10})$, $C_2F_3O_2$, O_{11}
formula weight	2565.00	2553.96
wavelength [Å]	0.939	0.939
crystal system	orthorhombic	hexagonal
space group	$P2_12_12_1$	$P6_5$
a [Å]	19.175(3)	28.781(2)
b [Å]	24.656(4)	28.781(2)
c [Å]	32.061(8)	34.911(2)
α [°]	90	90
β [°]	90	90
γ [°]	90	120
cell volume [Å^3]	15152(5)	25044(3)
ρ_{calcd} [g cm^{-3}]	1.124	1.016
Z	4	6
μ [mm^{-1}]	0.084	0.077
F(000)	5512	8214
crystal size [mm^3]	$0.29\times0.12\times0.12$	$0.32\times0.24\times0.15$
T [K]	100(2)	100(2)
θ range [°]	1.4–28.0	1.3–28.0
measured reflections	31070	39521
unique reflections	7530	8373
completeness [%]	88.0	93.7
redundancy	4.1	4.7
$R_{(merge)}$	0.058	0.065
data in refinement	7514	8342
data with $F_o > 4\sigma(F_o)$	6657	7721
average $\sigma(I)$	15.0	18.8
no. of parameters	1928	1811
extinction coefficient	0.032(3)	0.26(2)
$wR2$	0.3352	0.3856
$R1$ (obs data)	0.1415	0.1579
$R1$ (all data)	0.1344	0.1609
Gof = S	1.726	2.070
ΔF peak/hole [e Å^{-3}]	0.80/−0.47	0.40/−0.28

0021, lot nr. 900711, 2 g per 400 mL broth), in microtiter plates (96 wells). The peptides GS and **6–9** were dissolved in ethanol (4 g L^{-1}) and diluted in distilled water (1000 mg L^{-1}), and two-fold diluted in the broth (64, 32, 16, 8, 4 and 1 mg L^{-1}). The plates were incubated at 30 °C (24–96 h) and the MIC was determined as the lowest concentration inhibiting bacterial growth. The experiments were conducted once, the experimental error is one MIC interval (a factor two).

Hemolytic assays: Freshly drawn heparinized blood was centrifuged for 10 min at 1000 g at 10 °C. Subsequently, the erythrocyte pellet was washed three times with 0.85 % saline solution and diluted with saline to a 1/25 packed volume of red blood cells. The peptides to be evaluated (**6–9** and GS) were dissolved in a 30 % DMSO/0.5 mM saline solution to give a 1.5 mM solution of peptide. If a suspension was formed, the suspension was sonicated for a few seconds. A 1 % Triton-X solution was prepared. Subsequently, 100 μL of saline solution was dispensed in columns 1–11 of a microtiter plate, and 100 μL of 1 % Triton solution was dispensed in column 12. To wells A1–C1, 100 μL of the peptide was added and mixed properly. 100 μL of wells A1–C1 was dispensed into wells A2–C2. This process was repeated until wells A10–C10, followed by discarding 100 μL of wells A10–C10. These steps were repeated for the other peptides. Subsequently, 50 μL of the red blood cell solution was added to the wells and the plates were incubated at 37 °C for 4 h. After incubation, the plates were centrifuged at 1000 g at 10 °C for 4 min. In a new microtiter plate, 50 μL of the supernatant of each well was dispensed into a corresponding well. The absorbance at 405 nm was measured and the percentage of hemolysis was determined. Experiments were carried out in duplo. Multiple blood donors (both male and female) were used. A maximum of 10 % experimental error was found.

X-ray crystallography

Crystallization: Suitable colorless prism-shaped crystals were obtained after slow evaporation of 2 μL droplets of 8.8 mg mL^{-1} **7** in an 80 % solution of MeOH in H_2O plus 2 μL of 0.5 M NaOH in H_2O under paraffin oil in Terasaki plates. Colorless prism-shaped crystals were obtained after slow evaporation of 2 μL droplets of 7.0 mg mL^{-1} **8** in 50 % solution of MeOH in H_2O plus 2 μL of 0.5 M $CaCl_2$ in MeOH under paraffin oil in Terasaki plates.

Crystal structure determination of 7 and **8A** crystal was mounted in air and then rapidly transferred to liquid nitrogen. Synchrotron data were collected at beamline ID14-2 at the ESRF (Grenoble, France). Images were collected with DNA software,[40] processed with MOSFLM[41] and scaled with POINTLESS and SCALA.[42] Both structures could be solved by direct methods using the SHELXD[43] program and were refined by full-matrix least-squares methods on F^2 with SHELXL[42] included in the WinGX[44] package. All hydrogen positions were calculated and refined using a riding atom model. There are two crystallographically independent molecules per asymmetric unit in both crystal structures and there are several disordered parts in all molecules. All non-hydrogen atoms were refined using anisotropic refinement. Selected crystallographic data is reported in Table 3.

CCDC 751340 (**7**), 751341 (**8**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif

Acknowledgements

We are grateful to Kees Erkelens and Fons Lefeber for assistance in measuring NMR spectra. We thank Pablo Guardado-Calvo and Bruno Dacunha-Marinho for help with crystallographic software. This work was supported by a grant from the Leiden Institute of Chemistry and BFU2008-01588/BMC of the Spanish Ministry of Science and Innovation. J.M.O. is supported by an "Angeles Alvariño" contract of the Xunta de Galicia.

[1] G. F. Gause, M. G. Brazhnikova, *Nature* **1944**, *153*, 703.
[2] L. H. Kondejewski, S. W. Farmer, D. S. Wishart, R. E. W. Hancock, R. S. Hodges, *Int. J. Pept. Protein Res.* **1996**, *47*, 460–466.
[3] G. M. J. Schmidt, D. C. Hodgkin, B. M. Oughton, *Biochem. J.* **1957**, *65*, 744–750.
[4] E. J. Prenner, R. N. A. H. Lewis, R. N. McElhaney, *Biochim. Biophys. Acta Biomembr.* **1999**, *1462*, 201–221.
[5] A. Stern, W. A. Gibbons, L. C. Craig, *Proc. Natl. Acad. Sci. USA* **1968**, *61*, 734–741.
[6] S. E. Hull, R. Karlsson, P. Main, M. M. Woolfson, E. J. Dodson, *Nature* **1978**, *275*, 206–207.
[7] A. Liquori, P. De Santis, *Int. J. Biol. Macromol.* **1980**, *2*, 112–115.
[8] S. Rachovsky, H. A. Scheraga, *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 6965–6967.
[9] K. Sato, U. Nagai, *J. Chem. Soc. Perkin Trans. 1* **1986**, 1231–1234.
[10] A. C. Bach, J. A. Markwalder, W. C. Ripka, *Int. J. Pept. Protein Res.* **1991**, *38*, 314–323.
[11] S. Roy, H. G. Lombart, W. D. Lubell, R. E. W. Hancock, S. W. Farmer, *J. Pept. Res.* **2002**, *60*, 198–214.
[12] N. de La Figuera, I. Alkorta, M. T. Garcia-Lopez, R. Herranz, R. Gonzalez-Muniz, *Tetrahedron* **1995**, *51*, 7841–7856.
[13] W. C. Ripka, G. V. de Lucca, A. C. Bach, R. S. Pottorf, J. M. Blaney, *Tetrahedron* **1993**, *49*, 3609–3628.
[14] G. M. Grotenbreg, M. S. M. Timmer, A. L. Llamas-Saiz, M. Verdoes, G. A. van der Marel, M. J. van Raaij, H. S. Overkleeft, M. Overhand, *J. Am. Chem. Soc.* **2004**, *126*, 3444–3446.
[15] G. M. Grotenbreg, M. Kronemeijer, M. S. M. Timmer, F. El Oualid, R. M. van Well, M. Verdoes, E. Spalburg, P. A. V. van Hooft, A. J. de

Neeling, D. Noort, J. H. van Boom, G. A. van der Marel, H. S. Overkleeft, M. Overhand, *J. Org. Chem.* **2004**, *69*, 7851–7859.
[16] G. M. Grotenbreg, A. E. M. Buizert, A. L. Llamas-Saiz, E. Spalburg, P. A. V. van Hooft, A. J. de Neeling, D. Noort, M. J. van Raaij, G. A. van der Marel, H. S. Overkleeft. M. Overhand, *J. Am. Chem. Soc.* **2006**, *128*, 7559–7565.
[17] R. Wijtmans, M. K. S. Vink, H. E. Schoemaker, F. L. van Delft, R. H. Blaauw, F. P. J. T. Rutjes, *Synthesis* **2004**, 641–662.
[18] G. M. Grotenbreg, A. E. Christina, A. E. M. Buizert, G. A. van der Marel, H. S. Overkleeft, M. Overhand, *J. Org. Chem.* **2004**, *69*, 8331–8339.
[19] K. Wüthrich, in *NMR of proteins and nucleic acids*, Wiley-Interscience, New York, **1986**, pp. 162–175.
[20] D. S. Wishart, B. D. Sykes, F. M. Richards, *Biochemistry* **1992**, *31*, 1647–1651.
[21] A. J. Maynard, G. J. Sharman, M. S. Searle, *J. Am. Chem. Soc.* **1998**, *120*, 1996–2007.
[22] E. G. von Roedern, E. Lohof, G. Hessler, M. Hoffmann, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 10156–10167.
[23] C. Kühn, G. Lindeberg, A. Gogoll, A. Hallberg, B. Schmidt, *Tetrahedron* **1997**, *53*, 12497–12504.
[24] L. Szano, B. L. Smith, K. D. McReynolds, A. L. Parrill, E. R. Morris, J. Gervay, *J. Org. Chem.* **1998**, *63*, 1074–1078.
[25] M. D. Smith, T. D. W. Claridge, G. E. Tranter, M. S. P. Sansom, G. W. J. Fleet, *Chem. Commun.* **1998**, 2041–2042.
[26] T. K. Chakraborty, S. Javaprakash, P. V. Diwan, R. Nagarai, S. R. B. Jampani, A. C. Kunwar, *J. Am. Chem. Soc.* **1998**, *120*, 12962–12963.
[27] A. B. Smith III, S. Sasho, B. A. Barwis, P. Sprengeler, J. Barbosa, R. Hirshmann, B. S. Cooperman, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3133–3136.
[28] H. S. Overkleeft, S. H. L. Verhelst, E. Pieterman, W. J. Meeuwenoord, M. Overhand, L. H. Cohen, G. A. van der Marel, J. H. van Boom, *Tetrahedron Lett.* **1999**, *40*, 4103–4106.
[29] K. Brickmann, Z. Q. Yuan, I. Sethson, P. Somfai, J. Kihlberg, *Chem. Eur. J.* **1999**, *5*, 2241–2253.
[30] U. Koert, *J. Prakt. Chem./Chem.-Ztg.* **2000**, *342*, 325–333.
[31] R. M. van Well, H. S. Overkleeft, M. Overhand, E. V. Carstenen, G. A. van der Marel, J. H. van Boom, *Tetrahedron Lett.* **2000**, *41*, 9331–9335.
[32] E. Lohof, E. Planker, C. Mang, F. Burkhart, M. A. Haubner, H. J. Wester, M. Schwaiger, G. Hölzemann, S. L. Goodman, H. Kessler, *Angew. Chem.* **2000**, *112*, 2868–2871; *Angew. Chem. Int. Ed.* **2000**, *39*, 2761–2764.
[33] B. Aguilera, G. Siegal, H. S. Overkleeft, N. J. Meeuwenoord, F. P. J. T. Rutjes, J. C. M. van Hest, H. E. Schoemaker, G. A. van der Marel, J. H. van Boom, M. Overhand, *Eur. J. Org. Chem.* **2001**, *8*, 1541–1547.
[34] F. Schweizer, *Angew. Chem.* **2002**, *114*, 240–264; *Angew. Chem. Int. Ed.* **2002**, *41*, 230–253.
[35] S. A. W. Gruner, E. Locardi, E. Lohof, H. Kessler, *Chem. Rev.* **2002**, *102*, 491–514.
[36] T. K. Chakraborty, S. Ghosh, S. Javaprakash, *Curr. Med. Chem.* **2002**, *9*, 421–435.
[37] M. D. P. Risseeuw, M. Overhand, G. W. J. Fleet, M. I. Simone, *Tetrahedron: Asymmetry* **2007**, *18*, 2001–2010.
[38] S. Aravinda, V. V. Harini, N. Shamala, C. Das, P. Balaram, *Biochemistry* **2004**, *43*, 1832.
[39] A. L. Llamas-Saiz, G. M. Grotenbreg, M. Overhand, M. J. van Raaij, *Acta Crystallogr.* **2007**, *D63*, 401–407.
[40] A. G. W. Leslie, H. R. Powell, G. Winter, O. Svensson, D. Spruce, S. McSweeney, D. Love, S. Kinder, E. Duke, C. Nave, *Acta Crystallogr. Sect. D* **2002**, *58*, 1924–1928.
[41] A. G. W. Leslie, *Acta Crystallogr. Sect. D* **2006**, *62*, 48–57.
[42] P. Evans, *Acta Crystallogr. Sect. D* **2006**, *62*, 72–82.
[43] G. M. Sheldrick, *Acta Crystallogr. Sect. A* **2008**, *64*, 112–122.
[44] L. J. Farrugia, *J. Appl. Crystallogr.* **1999**, *32*, 837–838.

Received: October 28, 2009
Revised: December 21, 2009
Published online: March 5, 2010

